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I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at the putrescine N-methyltransferase (PMT) step and specifically to isolate PMT from tobacco root extracts.

B. **Status**: Preliminary studies were conducted to determine the change in the PMT activity of tobacco root harvested at various times after topping. Fifty-two Burley 21 tobacco plants from Group 17 were topped on 3/21/89. Thirteen of the 52 tobacco plants were harvested directly after topping for the T_0 sample. The remaining plants were divided into three groups of thirteen plants and harvested 24 hours, 48 hours, and 168 hours after topping. The specific activity of the roots after topping as compared to T_0 revealed no real changes for the T_{24} sample and increases of 2 and 3 fold for T_{48} and T_{168} respectively. Leaves (top, mid-stalk and bottom) harvested at various times after topping have been frozen at -80°C to determine if they contain any PMT. All the roots from group 17 of tobacco plants have been harvested and processed to obtain ammonium sulfate (40-65%) extracts (1).

Two 100 ml aliquots from a phenyl-Sepharose column (2) were processed through the S-adenosylhomocysteine (SAH) affinity matrix column (blocked with mercaptoethanol) for further purification of PMT. PMT was eluted from this column with 1 mM S-adenosylmethionine (SAM). The PMT active fractions from the SAH columns were stored at -80°C (3).

Various other PMT active samples and PMT active fractions which did not bind to various SAH columns were concentrated using a DEAE-Sepharose column. Active PMT fractions were bound to the column in dialysis buffer and eluted with 0.2 M NaCl in dialysis buffer (2). These samples were stored at -80°C for use with HPLC.

The sample concentrated by DEAE Sepharose was used to determine the molecular weight of PMT using a TosoHaas PWXL-4000 gel permeation column. An apparent molecular weight between 60 and 70 kD was derived for PMT as calculated by W. P. Hempfling (4). The previous reported MW based on analysis on a silica matrix was lower presumably due to the influence of the salt concentration (3,4).

Using the DEAE concentrated sample (3), a FPLC chromatofocusing column (HR5/5) was examined for fractionation of PMT. PMT activities were eluted at pH 5.1 and pH 5.2 using shallow and steep pH gradients, respectively (4). This is supportive of the earlier determination that isoelectric point of PMT is 5.1 ± 0.1. The Supelco (C-4 and LC Hint) hydrophobic interaction columns were also examined but deemed to be of little value since PMT activities were

spread over many fractions. However, a Poly LC polypropyl aspartamide hydrophobic interaction column appears promising. The PMT activity can be bound to the polypropyl aspartamide gel in presence of 1 M $(NH_4)_2SO_4$ and eluted with about 0.5 M $(NH_4)_2SO_4$ (3,4).

As compared to the 2 cm, the 1 cm diameter gel with ELFE looks promising for collecting active PMT fractions. Various parameters with ELFE have been examined. Maximum protein loading capacity on a 1 cm diameter ELFE gel is being determined. Simultaneous runs in which two identical ELFE's, except for the presence or absence of a stacking gel, revealed similar banding patterns of the active PMT fractions. However, the active PMT off the non-stacking gel ELFE eluted in fraction six while the active PMT with the stacking gel eluted in fraction sixteen. Furthermore, there was less tailing observed in the non-stacking gel (5).

The routine Bio Rad Assay was modified to determine protein concentrations in dilute samples. The modification made was to use a large sample volume so that more proteins will be present in the sample to be detected by the dye. The concentrated dye solution is diluted using the sample as the diluent instead of distilled water as in the routine method. Therefore, it is possible to increase the sample volume from 100 µl to 900 µl. Using the modified method, the standard curve constructed using gamma globulin agreed well with that obtained from the routine method. An SAH sample was used to evaluate the validity of the modified method. Accurate determination of the protein levels were seen for SAH samples diluted up to approximately 100 fold (4-5 µg/ml); however, when the SAH sample was diluted 200 fold to approximately 2-3 µg/ml, the determination was not accurate (6).

C. **Plans**: Continue to purify PMT samples from the ammonium sulfate extract through phenyl-Sepharose, SAH, and AEA columns. Implement further protein purification using an HPLC scheme. Explore various conditions using the Rotofor and native gel and ELFE systems. Complete proposal on differential hybridization. Continue to evaluate the modified Lowry method.

D. **References**:

1. Lyle, J. Notebook No. 8397.
2. Malik, V. Notebook No. 8724.
3. Mooz, E. Notebook No. 8599.
4. Nakatani, H. Notebook No. 8384.
5. Davies, S. Notebook No. 8761.
6. Yu, T. Notebook No. 8806.